The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi

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The hypothesis is advanced that NADP+-malic enzyme (ME; EC 1.1.1.40) is an important activity in regulating the extent of lipid accumulation in filamentous fungi. In Mucor circinelloides, a fungus capable of accumulating only 25% (w/w, dry wt) lipid, even under the most propitious conditions, ME disappears 15–20 h after nitrogen exhaustion, coincident with the cessation of lipid accumulation. In contrast, ME in Mortierella alpina, a fungus capable of accumulating 50% (w/w, dry wt) lipid, remains active for over 60 h after N-exhaustion during which time lipid accumulation continues. No other enzyme activity studied, including the lipogenic enzymes acetyl-CoA carboxylase, fatty acid synthase, diacylglycerol acyltransferase, ATP:citrate lyase and the NADPH-generating enzymes glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADP⁺:isocitrate dehydrogenase, demonstrated any correlation with the accumulation of storage lipid in either fungus. Full activity of ME is restored in Mr. circinelloides within 4 h by adding NH₄⁺ to the cultures, but this is prevented by adding cycloheximide as an inhibitor of protein synthesis. This suggests that the decrease in ME activity occurs due to down-regulation of the ME gene.

Keywords: lipids, malic enzyme, Mortierella alpina, Mucor circinelloides

INTRODUCTION

Many eukaryotic micro-organisms (yeast, fungi, algae) accumulate lipid as a storage material when growth becomes limited due to the exhaustion of the nitrogen source, or other nutrient, but where an excess of the carbon source remains (Ratledge, 1997). However, the extent of lipid accumulation differs widely between species, from as little as 3–5% (w/w, dry wt) to in excess of 80% (w/w, dry wt). ATP:citrate lyase (ACL), an enzyme involved in the provision of cytosolic acetyl-CoA for anabolic processes, including lipogenesis, has been found to be crucial in allowing the accumulation of substantive amounts of lipid (> 20%) in fungi (Botham & Ratledge, 1979; Evans & Ratledge, 1985). However, even in fungi possessing ACL, there exist considerable differences in the capacity to accumulate cell lipid between closely related species. As strains of yeast that possess ACL but do not accumulate storage lipid have been reported (Ratledge & Gilbert, 1985) it is evident that the biochemical reason for different species accumulating different amounts of lipid must lie elsewhere than the possession of this activity.

More recently the importance of malic enzyme (ME; EC 1.1.1.40) in the accumulation of storage lipid by fungi has been reported (Wynn & Ratledge, 1997; Wynn et al., 1997). ME catalyses the reaction

\[ \text{L-Malate + NADP}^+ \rightarrow \text{pyruvate + CO}_2 + \text{NADPH} \]

and is thought to play a key role in the provision of NADPH for both \textit{de novo} fatty acid biosynthesis and fatty acid desaturation. Inhibitors of ME, such as sesamol, lead to decreased lipid accumulation (Wynn et al., 1997) and mutants of \textit{Aspergillus nidulans} lacking ME activity also have diminished lipid contents (Wynn & Ratledge, 1997). This study examines the potential role of ME activity in the regulation of lipid accumulation in two oleaginous fungi – \textit{Mucor circinelloides}, which has been used commercially to produce an oil rich in γ-linolenic acid (Ratledge, 1997), which has an upper
limit for lipid accumulation of about 25% (w/w, dry wt) of the biomass, and Mortierella alpina, which is now being used to produce an oil rich in arachidonic acid, has an upper limit for lipid accumulation of about 25% (w/w, dry wt) of the biomass, and Mortierella alpina, which is now being used to produce an oil rich in arachidonic acid.

**METHODS**

**Chemicals.** Unless otherwise stated all fine chemicals were obtained from Sigma.

**Cultivation of fungi.** Mucor circinelloides CBS 108.16 and Mortierella alpina peyron CBS 696.70 were maintained on Kendrick medium plates (Kendrick & Ratledge, 1992) and stored at 4 °C. Fungal cultures were initially cultivated in 1 l stirred bottles containing 800 ml Kendrick medium. These cultures were incubated for 16 h at 30 °C then used to provide a 10% (v/v) seed to inoculate 4 l (working volume) fermenters containing modified Kendrick medium, ammonium tartrate (2 g l⁻¹) and glucose (50 and 30 g l⁻¹ for M. circinelloides and M. alpina, respectively). Fermenters were incubated at 30 °C, stirred at 500 r.p.m. with aeration at 0.5 v/v min⁻¹ and pH was maintained at 5.5 by automatic addition of KOH and HCl.

**Production of cell extracts.** Culture samples were removed periodically from the fermenters for analysis. Biomass was harvested by filtration (under reduced pressure) through a Whatman no. 1 filter and washed with cold purified water. Cell extracts for the determination of enzyme activities other than diacylglycerol acyltransferase (DAGAT) were prepared by suspending mycelia in an extraction buffer containing 100 mM KH₂PO₄/KOH (pH 7.5), 20% (w/v) glycerol, 1 mM benzamide/HCl and 1 mM dithiothreitol, and disrupted by passage twice through a French pressure cell at 35 MPa. Disrupted cell suspensions were centrifuged at 10000 g for 10 min and the supernatant retained for enzyme analysis. Extracts for the determination of DAGAT were prepared as described above but the extraction buffer contained 10 mM KH₂PO₄/KOH (pH 7.0), 250 mM sucrose and 1 mM EDTA. Protein concentrations were determined using the method of Bradford (1976) with BSA as a standard.

**Estimation of enzyme activities.** The activities of malic enzyme (ME; EC 1.1.1.40), glucose-6-phosphate dehydrogenase (G-6-PDH; EC 1.1.1.49), 6-phosphogluconate dehydrogenase (6-PGDH; EC 1.1.1.44), NADP⁺-isocitrate dehydrogenase (NADP⁺:ICDH; EC 1.1.1.42), fatty acid synthase (FAS; EC 2.3.1.86) and ATP:citrate lyase (ACL; EC 4.1.3.8) were assayed as described by Wynn et al. (1997). Acetyl-CoA carboxylase (ACC; EC 6.4.1.2) was assayed as described by Matthews et al. (1990). Diacylglycerol acyltransferase (DAGAT; EC 2.3.1.20) activity was determined using the assay described by Kamisaka et al. (1993) except that the solvent system used for separating the reaction products was petroleum spirit (40°-60°)/diethylether/formic acid (85:15:1, by vol.) and radioactive spots were quantified using a Bioscan System 400 Imaging TLC plate scanner. All assays were carried out at 30 °C.

**Determination of culture dry weight.** A 20 ml sample of the culture was harvested onto a washed, dried and weighed filter (Whatman no. 1). The filtrate was retained and analysed for culture glucose and ammonium concentrations (see below). Harvested biomass was washed with distilled water and then dried at 100 °C to constant weight. The weight of the biomass was determined gravimetrically.

**Analysis of culture supernatant.** The glucose concentration in the culture medium was determined using a glucose oxidase Perid-Test kit (Boehringer Mannheim), according to the manufacturer’s instructions. The ammonium concentration in the culture filtrate was determined using the indophenol test (Chaney & Marbach, 1962).

**Analysis of cell lipid.** Fungal biomass was harvested by filtration (Whatman no. 1), washed with distilled water and freeze-dried. Cell lipid was extracted with 2:1 (v/v) chloroform/methanol (Folch et al., 1957) and determined gravimetrically.

**Reproducibility of data.** All experiments were carried out at least three times, except the experiments to restore ME activity by addition of NH₄⁺ which were carried out in duplicate. All data presented are reproducible; however, typical data rather than mean data are presented due to some inter-experiment variation in the time of NH₄⁺ exhaustion in the fermenters.
RESULTS AND DISCUSSION

Growth and lipid accumulation in *Mr. circinelloides* and *Mt. alpina*

Both fungi demonstrated a similar, and typical, growth profile in batch fermentation (Fig. 1). Growth was initially rapid during the nitrogen utilization phase, after which growth slowed, although a small increase in biomass continued, presumably due to the accumulation of storage lipid and hyphal wall thickening.

Lipid accumulated after the exhaustion of the nitrogen source. Initially the rate of lipid accumulation was not dissimilar in the two fungi: at 40 h both contained 20–25% (w/w, dry wt) lipid. However, whilst in *Mr.*...
The extent of lipogenesis. This observation is in accord with those of Kang et al., 1994, is unlikely to be important in determining the extent of lipogenesis. In this regard, ACC, although probably an enzyme with a regulatory role governing the rate of lipid synthesis in fungi, as has been suggested in plants (Page et al., 1994), was in accord with those of Kang et al. (1994) and Roesler et al. (1997) in plant systems.

**Activities of key enzymes during lipid accumulation in *Mr. circinelloides* and *Mt. alpina***

A range of enzymes involved, or potentially involved, in the biochemistry of oleaginicity were studied in both fungi. These enzymes included those belonging to the lipid biosynthetic pathway itself, ACC, FAS and DAGAT; ACL, which is implicated in the production of cytosolic acetyl-CoA for lipid synthesis in oleaginous yeasts (Botham & Ratledge, 1979; Evans & Ratledge, 1985); and a range of enzymes potentially involved in the generation of NADPH for fatty acid synthesis, ME, G-6-PDH, 6-PGDH and NADP⁺:ICDH.

Although the specific activities of the enzymes varied to some degree between the two fungi, the developmental profiles for all but one of the enzymes were similar (Fig. 2), the exception being ME which is discussed below. Activities of the other enzymes studied were detectable many hours after nitrogen exhaustion and, for *Mr. circinelloides*, at a time when lipid accumulation had ceased. There was, however, a decline in the activity of some enzymes toward the end of growth, notably ACL and NADP⁺:ICDH (see Fig. 2). FAS (Fig. 2a) though remained active even long after lipid accumulation had ceased in *Mr. circinelloides* and had virtually stopped in *Mt. alpina*. Similarly, DAGAT, which is responsible for the final reaction in the synthesis of the storage triacylglycerols, remained active in both fungi 24 h after nitrogen exhaustion (see Table 1).

The enzyme activity that demonstrated a different developmental profile between *Mr. circinelloides* and *Mt. alpina* was ME. In *Mr. circinelloides* the activity of ME was low [20–25 nmol min⁻¹ (mg protein)⁻¹] and decreased relatively rapidly after nitrogen exhaustion, becoming undetectable at 45 h (i.e. 25 h after NH₄⁺ exhaustion. In contrast, the activity of ME in *Mt. alpina* was initially higher [100–110 nmol min⁻¹ (mg protein)⁻¹] and, although its activity decreased after nitrogen exhaustion, it remained active even after 88 h. As equally important is the disappearance of ME activity in *Mr. circinelloides* was the timing of its disappearance. In *Mr. circinelloides*, ME activity became undetectable at 45 h, coincident with the cessation of lipid accumulation (Fig. 1a). At this time the lipogenic enzymes (ACL, FAS, ACC and DAGAT) and other providers of NADPH (G-6-PDH, 6-PGDH and NADP⁺:ICDH) all remained active (Fig. 2 and Table 1).

In *Mr. circinelloides*, therefore, lipid accumulation appears to be limited by a lack of NADPH for lipid synthesis associated with the disappearance of ME. Although our previous work has shown the importance of ME in the provision of NADPH for FAS activity, both in *Mr. circinelloides* and Aspergillus nidulans (Wynn & Ratledge, 1997; Wynn et al., 1997), this is the first indication that ME activity may play a crucial role in determining the extent of lipid accumulation in filamentous fungi.

In *Mt. alpina* ME activity remained detectable for in excess of 80 h (Fig. 1b) and this correlated not only with the prolonged period of lipid synthesis in this organism but, as the activity of ME itself began to decline late in the fermentation, with the decline in the rate of lipid accumulation. This slowing in lipid accumulation in *Mt. alpina* late in the fermentation when ME activity is in decline suggested that loss of ME activity eventually limited lipid accumulation in this organism too.

The reintroduction of nitrogen (NH₄⁺) to the medium was used to demonstrate that the disappearance of ME in *Mr. circinelloides* was in response to the exhaustion of the nitrogen source. This resulted in full activity of ME being restored within 4 h (see Fig. 3). De novo protein synthesis was required for the reinstatement of activity after the addition of NH₄⁺ as the addition of cycloheximide (100 µg ml⁻¹) (a known inhibitor of eukaryotic ribosome function) simultaneously with the introduction of NH₄⁺ prevented the recovery of ME.
activity. It was demonstrated that the effect of cycloheximide was not due to it inhibiting ME by adding cycloheximide to a culture replete with ME activity: under these conditions the activity of ME was not adversely affected. Nor did cycloheximide affect the in vitro assay of ME. Furthermore, the possibility that the disappearance of ME activity after nitrogen exhaustion was the result of the production of an inhibitor was precluded by the addition of a cell-free extract (produced after nitrogen limitation) devoid of ME activity to an extract containing ME activity: no diminution of activity was observed.

**DISCUSSION**

Many cells accumulate lipid, from simple eukaryotic yeasts and fungi to seed plants and animal cells (such as adipocytes). The extent to which these cells can accumulate lipid varies enormously with different, but sometimes closely related, species having large differences in their lipogenic capacity. No hypothesis has yet been advanced that can account for these differences though some researchers have suggested that the first committed enzyme of fatty acid synthesis, ACC, could well be the enzyme that controls the rate of lipid synthesis (Page et al., 1994), although even this is a matter of some controversy and doubt (Kang et al., 1994; Roesler et al., 1997). There is, however, no indication that ACC regulates the extent of lipid accumulation in cells. Furthermore, it is clear that the provision of acetyl-CoA itself, as the essential C2 building unit for fatty acid biosynthesis, is not limiting the extent of lipid accumulation. Acetyl-CoA is produced in oleaginous micro-organisms via ACL (Boulton & Ratledge, 1981a) which could also be the route of acetyl-CoA formation in plants (Ratledge et al., 1997) but, as shown both in this study and in previous work in this laboratory (Boulton & Ratledge, 1981a, b; Evans & Ratledge, 1985), ACL activity does not correlate with the extent of lipid accumulation in oleaginous microorganisms. The possibility that DAGAT activity, as the final step in triacylglycerol assembly, plays a key role in the regulation of lipid accumulation has likewise been precluded. In this study, DAGAT activity demonstrated no obvious correlation to the period of active lipid accumulation. This observation is in accord with previous observations in Mortierella ramanniana where DAGAT activity was detectable even after the cessation of lipid accumulation (Kamisaka et al., 1993).

ME appears, at least in the two fungi examined here, to be important in the provision of NADPH for lipid biosynthesis in vivo. None of the other NADPH-generating enzymes can apparently duplicate this role. Disappearance of ME activity thus appears to cause the cessation of lipid accumulation. No other enzyme activity that we have monitored here, or previously, has shown such a strong correlation with the extent of lipid accumulation.

In light of these findings we suggest that the key factor controlling the extent of lipid accumulation in oleagi-

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**Fig. 3.** Reinstatement of ME activity in *Mr. circinelloides* by the addition of ammonium tartrate (to a final concentration of 2 g l\(^{-1}\)). The reinstatement is possible both immediately after its initial disappearance (a) and 20 h after the disappearance of activity (b). The addition of cycloheximide at the same time as the reintroduction of nitrogen to the culture prevents the reinstatement of ME activity in *Mr. circinelloides* (c). The time of addition of ammonium tartrate to the culture is indicated by a down-pointing arrow.
ous micro-organisms is not the supply of carbon but the supply of H equivalents. It has always been assumed that reducing equivalents for biosynthetic purposes could be generated by any NADPH-producing enzyme: G-6-PDH, 6-PGDH, NADP⁺:ICDH or ME. The assumption was that there exists a common pool of NADPH from which any NADPH-requiring enzyme could draw its supply of co-factor, irrespective of the status of the cell. The data obtained in this study, along with our previous published work (Kendrick & Ratledge, 1992; Wynn et al., 1997; Wynn & Ratledge, 1997), suggest an important role for ME specifically in the provision of NADPH for lipid synthesis. These observations are not consistent with the existence of a generally available cytosolic ‘pool’ of NADPH, at least for lipogenesis.

The manner in which ME is integrated into fatty acid biosynthesis remains to be determined. However the ‘metabolon’ concept advanced by Sere (1987), who showed a physical association between the lipogenic enzymes ACL, ACC and FAS in animal cells, may be one explanation. If a lipogenic metabolon does indeed exist we would suggest that it is likely to include ME as a provider of NADPH. A physical association between ME and FAS, leading to the channelling of NADPH between the active sites of these two enzymes is, we suggest, the most likely explanation for the apparent importance of ME in the lipogenic process.

The regulation of ME appears to be exerted at a level prior to protein translation as shown by the failure of ME activity to recover in cells treated with cycloheximide. We would suggest that the likely site of regulation of ME activity is at the level of gene expression, although we cannot rule out the possibility that RNA processing plays a role.

Whatever the exact mechanism of regulating ME activity, the identification of this enzyme activity as a potentially important factor in controlling lipid accumulation in cells means that ME may represent an attractive target to those attempting to alter the lipogenic potential of microbial cells. These findings are therefore of importance to those attempting to increase the productivity of microbial cells for the commercial production of single cell oils in which the extent of lipid accumulation could perhaps be increased by up-regulation of the ME gene. Conversely, in micro-organisms synthesizing other secondary metabolites from acetyl-CoA – such as terpenes and polyketides – the formation of triacylglycerols, which represents a wasteful biosynthetic pathway competing for the carbon supply, could be curtailed by down-regulation of the gene encoding ME.

Furthermore, if the generality of these results can be confirmed for other eukaryotic cells then in plants an increase in seed oil accumulation may also be possible by manipulation of the ME gene. Indeed a key role for ME in the regulation of plant lipogenesis has already been suggested (Colombo et al., 1997).

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